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(57) Abstract

The present invention provides a method of treating a neoplastic cell comprising administering a pharmacologically effective dose of actinonin to said cell. Preferably, actinonin can be used to treat neoplastic cell such as lymphomas, leukemias, carinomas, sarcomas, and other pathological states in humans involving lymphocytes such as infections and auto-immune disorders.

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ANTI-NEOPLASTIC EFFECTS OF ACTINONIN

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the fields of immunology and cancer treatment. More specifically, the present invention relates to anti-neoplastic effects of actinonin.

Description of the Related Art

CD13/Aminopeptidase-N (EC 3.4.11.2) is a ubiquitous cell surface zinc aminopeptidase involved in down regulation of regulatory peptide signals (1). Recently, aminopeptidase N has been shown to be the major receptor for the enteropathogenic coronavirus TGEV (2) and for human coronavirus 229E (3), and to be involved in tumor-cell invasion (4, 5). Human aminopeptidase N is identical to the myeloid differentiation antigen CD13 (6, 7), found on HL60 leukemia cells (7, 8), myeloid and monocytic cells and most myeloblastic leukemias, as well as on cells and tissues outside the

hematopoietic system including fibroblasts, intestinal epithelium, renal tubular epithelium and synaptic membranes of the central nervous system (1). Aminopeptidase N occurs as a homodimer and the molecule is a 150-kDa, transmembrane glycoprotein with an intracellular amino terminus (1). F23, an anti-human CD13/aminopeptidase N monoclonal antibody, is able to completely block the active site of the enzyme (9).

Bestatin, a CD13/aminopeptidase N inhibitor, has been examined in preclinical and clinical studies. Bestatin inhibited lymph node metastasis of P388 leukemia in mice (10), and was used in clinical trials in malignant skin tumors (11), in head and neck cancer (12), in esophageal cancer (13), and in gynecologic tumors (14). High doses of bestatin resulted in the significant inhibition of preexisting experimental and spontaneous metastasis in mice (15).

Actinonin, (3-[[1-2-(hydroxymethyl)-pyroli-dinyl] carbony]-2-methyl-propyl]carbamoyl]octanohydroxaminic acid, a naturally occurring antibiotic derivative of L-prolinol and a potent CD13/aminopeptidase N inhibitor, is obtained from the culture filtrates of a *Streptomyces* species classified as *Streptomyces* Cutter C/2 NCIB 8845 (16). Actinonin has been shown to be generally active against Gram-positive bacteria. The action of the antibiotic involves disruption of RNA synthesis in bacteria. *In vivo* , it has no apparent toxicity to mice in doses up to 400 mg/kg body weight (16). Actinonin is eight times more potent than bestatin (9).

The prior art is deficient in the lack of the demonstration of anti-tumor and cytostatic activity of actinonin *in vivo* and effective means of treating tumors using

actinonin. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The present invention discloses an anti-cancer agent, actinonin, which has anti-proliferative effects on human leukemia and lymphoma cells *in vitro* and on syngeneic leukemia *in vivo*. In one embodiment of the present invention, there is provided a method of treating a neoplastic cell comprising administering a pharmacologically effective dose of actinonin to the cell. Preferably, actinonin can be used to treat neoplastic cell such as lymphomas, leukemias, and carcinomas, other pathological states in humans involving lymphocytes such as infections and auto-immune disorders. Generally, actinonin has anti-proliferative activity on tumors both *in vitro* and *in vivo* by inducing cell growth arrest at G1 and inducing cell apoptosis.

In one embodiment of the present invention, there is provided a method of treating an individual having a lymphoma, comprising the step of administering a therapeutically effective dose of actinonin to the individual.

In one embodiment of the present invention, there is provided a method of treating an individual having a leukemia, comprising the step of administering a therapeutically effective dose of actinonin to the individual.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments

of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows cytotoxicity and inhibition of protein synthesis in cell lines by actinonin. **Figure 1A** shows inhibition of protein synthesis in cells by actinonin. Cell lines ($5x10^4$ cells/ml) were incubated for 5 days at 37°C in the presence of actinonin. Levels of protein synthesis were determined by a 5 hour incorporation of tritiated leucine into trichloroacetic acid-precipitable protein. **Figure 1B** shows cell viability determined by trypan blue exclusion. Cell lines ($5x10^4$ cells/ml) were incubated for 5 days at 37°C in the presence of actinonin. Trypan blue was added, and live and dead cells were enumerated.

Figure 2 shows the effects of actinonin on the cell cycle. Aliquots of HL60 cells were collected at different times after actinonin treatment (10 µg/ml).

Figure 3 shows effects of actinonin on AKR leukemia cells *in vivo*. **Figure 3A** shows survival curves of AKR mice after transplantation of AKR leukemia cells. AKR leukemia cells ($2x10^6$) were transplanted into AKR mice. Three days after the transplantation, the mice were treated with actinonin intraperitoneally, daily for 5 days. **Figure 3B** shows treatment of AKR leukemia cells *in vivo* by actinonin. AKR mice were transplanted subcutaneously with $2x10^6$ AKR leukemia cells. After the third day, mice were treated intraperitoneally with 100 µg actinonin per mouse, daily for 3 days, then treated with an additional injections of 100 µg actinonin (one injection of 100 µg actinonin every other day for three times). At the times indicated on the X axis, tumor surface area (mm^2) was measured.

DETAILED DESCRIPTION OF THE INVENTION

Actinonin, an antibiotic and CD13/aminopeptidase-N inhibitor, has been shown to be cytotoxic to tumor cell lines *in vitro*. The present invention details the anti-proliferative effects of actinonin on human and murine leukemia and lymphoma cells. Also disclosed is a method of treating an individual with actinonin.

Thus, the present invention is directed a method of treating a neoplastic cell comprising administering a pharmacologically effective dose of actinonin to the cell. Representative examples of neoplastic cells include lymphomas, leukemias, carinomas, sarcomas, and other pathological

states in humans involving lymphocytes such as infections and auto-immune disorders. Actinonin can be used against a neoplastic cell in a human or animal, for example, inducing cell growth arrest and apoptosis. Actinonin can be used to treat a neoplastic cell *in vitro*. Preferably, actinonin is administered to an individual at the concentration of about 1 mg/kg to about 100 mg/kg, in single or repeated doses.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Materials

Actinonin, amastatin and bestatin were purchased from Sigma (St. Louis, MO). Monoclonal antibodies 4B4, OKT9, Leu-11a, Leu-15, Leu-M1, MY4, control IgG1 and IgG2a were purchased from Coulter (Hialeah, FL) or Becton Dickinson (San Jose, CA). Fluoresceinated, affinity-purified goat antiserum to mouse immunoglobulins (GAM-FITC) were purchased from Kirkegaard & Perry (Gaithersburg, MD). Monoclonal antibodies F23, TA99, JD12 and M195 were produced in Memorial Sloan-Kettering Cancer Center. Recombinant human IL-3 was obtained from Amgen (Thousand Oaks, CA). Stock vials of human IL-3 was stored at 4°C. For each experiment, all factors were diluted in serum-containing medium on the day of use.

EXAMPLE 2

Cell Separation Techniques

Bone marrow cells were obtained from healthy volunteers after informed consent. The mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients (1.077 g/ml; Pharmacia Fine Chemicals, Piscataway, NJ), washed twice in phosphate-buffered saline (PBS), and suspended in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT) supplemented with penicillin (100 U/ml; GIBCO, Grand Island, NY), streptomycin (100 µg/ml; GIBCO), and 3 mg/ml glutamine (GIBCO). These cells were used as target cell populations for the colony forming unit-granulocyte macrophage (CFU-GM) progenitor cell assay.

EXAMPLE 3

Colony Forming Unit-Granulocyte Macrophage (CFU-GM)

Low-density ($1x10^5$ cells/ml) or $CD34^+$ ($5x10^3$ cells/ml) bone marrow cells were cultured in 35-mm tissue culture dishes (Corning, Corning, NY) in McCoy's modified assay medium containing 0.3% agar (DIFCO, Detroit, MI) and 10% FCS (17). Cultures were stimulated by the addition of 100 ng/ml IL-3.

EXAMPLE 4

Animals

Five-week-old female AKR mice were purchased from Jackson Laboratory (Bar Harbor, ME). All bedding

material was sterilized before use; the cages were covered with an air filter and maintained in isolation cabinets. Animal handling and experiments were performed in a sterile atmosphere using a laminar flow hood following institutional care guidelines.

EXAMPLE 5

Cell Lines and Culture Conditions

AKR leukemia cells were obtained from the spleen of old AKR mice who spontaneously developed leukemia at 10 months. HL60 (acute myeloid leukemia, CD13 positive), NB4 (acute promyelocytic leukemia, CD13 positive, from Dr. M. Lanotte of the Louis Pasteur Institute, Paris, France), RAJI and DAUDI (B lineage Burkitt's lymphomas, CD13 negative) were maintained in culture using RPMI 1640 supplemented with 10% Serum Plus (JRH Biosciences, Lenexa, KS) and 10% heat inactivated fetal calf serum (Intergen, Purchase, NY) at 37°C in a humidified atmosphere of 5% CO2 air. Cell viability was always higher than 90% and cells were free of mycoplasma contamination.

EXAMPLE 6

Transplantation of AKR Leukemia Cells into AKR Mice and Therapy

A 0.1 ml aliquot containing $2x10^6$ AKR leukemia cells from suspension culture was transplanted

subcutaneously into AKR mice. Tumors grew subcutaneously and the cutaneous tumor size was measured as a cross product to derive surface area. To protect animals, mice were considered "dead" and sacrificed when the tumor surface area reached more than 400 mm^2. The test animals were treated intraperitoneally with actinonin in a final volume of 0.1 ml. Control mice were treated with 0.1 ml saline.

EXAMPLE 7

Flow Cytometry Assays

Cells were washed and resuspended in 2% rabbit serum (Pel Freeze, Rogers, AK) to reduce nonspecific binding. $5x10^5$ cells in a final volume of 0.1 ml were incubated one hour on ice in the presence of primary antibody. Cells were washed twice, incubated 30 min on ice with secondary fluorescein isothiocyanate (FITC) labeled antibody (goat anti-mouse immunoglobulin) (Kirkegaard & Perry, Gaithersburg, MD), washed twice, and fixed with 0.5% paraformaldehyde. FITC fluorescence intensity was measured on an EPICS Profile II flow cytometer (Coulter, Hialeah, FL).

EXAMPLE 8

Inhibition of Tritiated Thymidine or Leucine Incorporation

An aliquot containing 200 µl of cells were washed and incubated at 37°C in 96 well plates in the presence or absence of actinonin. After an incubation time of 3 to 7

days, 50 µl of 10 µCi/ml of tritiated thymidine or leucine (Du Pont-New England Nuclear, Wilmington, DE) was added to each well and allowed to incorporate for 5 to 6 hours. Trichloroacetic acid was added at a final concentration of 10% to precipitate protein for [^{3}H]leucine incorporation experiments. Cells were harvested using a semiautomatic harvester (Skatron, Norway) and read in a scintillation counter LS 6000 IC (Beckman, Fullerton, CA).

EXAMPLE 9

Flow Cytometric Analysis of CD11b and BCL-2 Proteins

Cells were incubated for 1 hour on ice with phycoerythrin-conjugated anti-CD11b monoclonal antibody (Becton Dickinson, San Jose, CA) and then washed with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde for 10 min, and then exposed to 0.1% Triton-X100 for 10 min. After washing with PBS and blocked with 1% human AB serum, cells were incubated with FITC-conjugated anti-BCL-2 monoclonal antibody (DAKO A/S, Denmark) on ice for 30 min and then analyzed on an EPICS Profile II flow cytometer (Coulter, Hialeah, FL). Ten thousand events were counted for each sample. Mean peak fluorescence intensity (MPF) for an isotype-matched control antibody was set at 1.

EXAMPLE 10

Cell Cycle and Apoptosis Analysis by Flow Cytometry

Cells were collected and fixed in 1.5%

paraformaldehyde/PBS for 15 min. After washing with PBS, the cells were resuspended in 70% ice-cold ethanol and kept at -20°C for up to 5 days. Analysis for cell cycle distribution and apoptosis was performed according to the instructions in the APOPTAG kit (ONCOR, Gaithersburg, MD). Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA); evidence of apoptosis and percentage of cells in each phase of the cell cycle were analyzed by the CellFIT and PC-LYSIS software (Becton Dickinson, San Jose, CA).

EXAMPLE 11

Actinonin Inhibited Growth of Leukemia Cells *in vitro*.

Actinonin was tested for its ability to kill CD13 positive and CD13 negative cells. Activity and cytotoxicity were determined by inhibition of incorporation of [^{3}H]leucine into protein and by trypan blue exclusion. Dose-response curves were generated by testing the inhibitory effects of actinonin on the protein synthesis of NB4 and HL60 cells (CD13 positive) or RAJI (CD13 negative) in culture (see Figure 1A). In these *in vitro* studies, 2-5 μg/ml of actinonin was required to inhibit protein synthesis by 50% in CD13 positive and negative cells.

The cytotoxicity of actinonin was initially determined by trypan blue exclusion (Figure 1B and Table 1). The IC_{50} (the concentration of actinonin required to kill 50% of cells) ranged from 2 to 5 μg/ml, comparable to the concentration of actinonin required to inhibit 50% of protein synthesis. The similar dose-response curves for cytotoxicity and protein

synthesis inhibition on cells that expressed or did not express CD13/aminopeptidase N suggested that the mechanism of cytotoxicity did not necessarily involve inhibition of CD13/aminopeptidase N by actinonin. Treatment of NB4 and HL60 cells for 4 days with 100 µg/ml monoclonal antibody F23, which blocks substrate binding to CD13/aminopeptidase N and its activity (18), had no effect on cell viability (91% alive) in comparison to no treatment (90% alive) or an isotype matched control antibody TA99 (91% alive) (data not shown). In addition, cytotoxicity of actinonin was not abrogated by pretreatment of cells with F23 antibody, which blocks actinonin binding to its active site (20). These data together showed that the inhibition of cell growth by actinonin is not through the inactivation of cell surface enzyme.

TABLE 1

Effect of actinonin on cell viability determined by trypan blue exclusion *in vitro*.

Cell Type	IC_{50} (µg/ml) (Mean ± SD)*
HL60 cells	2 ± 0.5
NB4 cells	5 ± 1.0
DAUDI cells	2 ± 0.5
RAJI cells	4 ± 1.0
AKR cells	3 ± 0.6

*IC_{50} is the concentration of actinonin required to kill 50% of cells.

EXAMPLE 12

Cell Cycle G1 Arrest and Apoptosis in Leukemia Cells during Actinonin Treatment

Cell cycle distribution of leukemia cells showed small changes early after treatment with 10 µg/ml actinonin. After 24 hours of exposure to actinonin the number of HL60 cells arrested in G1 phase increased and the percentage of cells in S phase decreased (Figure 2). A similar effect was seen in NB4 (G1 phase increased by 24%) and RAJI (G1 phase increased by 37%) after 24 hours of treatment with 10 µg/ml actinonin (not shown). This G1 arrest is in accordance with the growth inhibition observed after 2 days of exposure to actinonin (not shown). 96 hours following exposure to actinonin at 10 µg/ml, 20-35% of HL60 and NB4 cells showed apoptosis, whereas only 10% of RAJI had apoptosis after treatment (Table 2).

TABLE 2

Effect of actinonin (10 µg/ml) on apoptosis in cells at 96 hours.

	HL60	NB4	RAJI
Control	2.9%	9.1%	4.3%
Actinonin	37.9%	29.4%	13.6%

EXAMPLE 13

Effects of Actinonin on AKR Leukemia Cells *in vivo*

The significant anti-proliferative effects of actinonin *in vitro* prompted an analysis of the effects of actinonin *in vivo* against leukemia or lymphoma cells. In order to avoid the problems associated with a xenograft model, a syngeneic leukemia/lymphoma model in AKR mice was used to better approximate actual human use. AKR cells are inhibited *in vitro* by actinonin with an IC_{50} of about 3 µg/ml (Table 1). In these experiments, AKR mice were injected subcutaneously with 2,000,000 AKR leukemia cells (day 0) and treated intraperitoneally with injections of actinonin beginning at day 3.

The effect of actinonin on tumor growth and survival rates of AKR mice after transplantation of AKR leukemia cells was investigated. After transplantation, mice were treated with a total of five injections of actinonin (one injection, daily for 5 days). As compared to controls, actinonin increased the mean survival time of mice by nearly twofold by reducing the rate of tumor growth. Reduction in tumor growth rates and prolongation of survival was actinonin dose-related (Figure 3A). Toxicity due to actinonin was not observed. Actinonin doses up to 8000 µg per mouse (400 mg/Kg) were tolerated without apparent toxicity (16).

In a second trial, mice were treated with 100 µg actinonin daily for 3 days beginning at day 3 after transplantation, then treated with an additional three injections of actinonin (every other day). On day 17, the control mice

showed tumors with a mean surface area of 287 ± 95 mm^2. In contrast, no tumors were found in mice in the actinonin treated group (Figure 3B). These results indicate that actinonin has significant anti-tumor effects on AKR leukemia *in vivo*. In contrast, actinonin did not have any significant growth inhibiting properties for subcutaneously implanted RAJI lymphoma in nude mice (data not shown) over the same dose range as was effective in the AKR model.

EXAMPLE 14

Effects of Actinonin on Human Bone Marrow Cell Growth *ex vivo*

The effects of actinonin, amastatin and bestatin were evaluated on normal human bone marrow colony forming unit-granulocyte macrophage (CFU-GM) (Table 3). Actinonin decreased 16-56% colony formation in a dose dependent manner. Bestatin (10 µg/ml) at doses designed to approximate its dose level in clinical trials (19) also decreased normal human bone marrow CFU-GM by 64-70%. This suggested that actinonin may have some myelosuppressive activities at high doses. F23 (anti-active site of CD13) and M195 (anti-CD33) both of which bind to bone marrow progenitors had no effect on human bone marrow CFU-GM on Day 7 and 14 (data not shown). These data further support the contention that the cytotoxic effects of actinonin are not mediated through CD13/aminopeptidase N.

TABLE 3

Response of low-density bone marrow cells to actinonin, amastatin and bestatin

Drugs (doses)	no IL-3	with IL-3: Number of colonies	
		Day 7	Day 14
		(% of inhibition)	(% of inhibition)
Control (saline)	0	177 ± 28	100 ± 4
Actinonin (10 µg/ml)	0	99 ± 18 (44%)	44 ± 8 (56%)
Actinonin (5 µg/ml)	0	123 ± 10 (31%)	49 ± 7 (51%)
Actinonin (0.5 µg/ml)	0	148 ± 6 (16%)	74 ± 7 (26%)
Amastatin (5 µg/ml)	0	157 ± 1 (11%)	82 ±-11(18%)
Bestatin (10 µg/ml)	0	63 ± 27 (64%)	30 + 7 (70%)

Data represent the mean ± standard deviation for four plates scored on day 7 and day 14. For these studies, $1x10^5$ low-density bone marrow cells were cultured for 7 and 14 days, respectively with IL-3 100 ng/ml.

EXAMPLE 15

Effects of Actinonin on BCL-2 Expression

BCL-2 expression was studied in preliminary experiments designed to screen for mechanisms or pathways involved in the anti-proliferative effects of actinonin. BCL-2 expression, observed by intracellular flow cytometry in about 20% of HL60 cells and 60% of NB4 cells, showed no significant changes after treatment with actinonin (Table 4). BCL-2

expression, however, was decreased by 80% in RAJI cells after treatment with actinonin.

TABLE 4

Effects of actinonin (10 µg/ml) on Bcl-2 expression in cell lines at 96 hours

	HL60	NB4	RAJI
Control	20.4%/1.7	58.8%/5.3	35.5%/3.1
Actinonin	18.5%/2.0	57.3%/5.1	8.0%/1.1

Data represent percentage of positive cells/MPF (mean peak fluorescence) intensity, i.e., protein density per cell.

EXAMPLE 16

Effects of Actinonin on Selected Cell Surface Protein Expression

The effects of actinonin were evaluated on selected cell surface protein expression to screen for mechanisms or pathways involved in the anti-proliferative effects of actinonin (Table 5). Cell surface proteins (CD11b, 13, 15, 29, 33, HLA-A) significantly decreased in NB4 cells after treatment with actinonin, consistent with the toxic effects of actinonin.

TABLE 5

Selected cell surface protein expression in NB4 cells *in vitro* after treatment with actinonin (10 μg/ml) for 48 hours.

Monoclonal Antibodies	- Actinonin	+ Actinonin
Control (IgG2a)	-/1	-/1
Control (IgG1)	-/1	-/1
Anti-HLA class I (JD12)	99.8/13.3	
	63.8/7.5	
Anti-CD13 (F23)	100/225.3	
	99.1/44.0	
Anti-CD29 (4B4)	100/46.8	
	90.9/10.8	
Anti-CD33 (M195)	99.6/12.6	
	22.1/3.3	
Anti-CD71 (OKT9)	97.7/11.3	
	79.1/11.4	
Anti-CD16 (Leu-11a)	-/1	-/1
Anti-CD11b (Leu-15)	64.8/2.35	
	6.2/1.43	
Anti-CD14 (MY4)	-/1	5.0/1
Anti-CD15 (Leu-M1)	99.5/129.088.6/25.0	

Data represent percentage of positive cells / MPF*.

*MPF is mean peak fluorescence intensity (protein density per cell).

Discussion

Actinonin, a naturally occurring derivative of L-prolinol, is a potent inhibitor of CD13/aminopeptidase-N (APN). The present invention shows 1) the anti-proliferative effects of actinonin on human leukemias and lymphoma cells *in vitro*, 2) the induction of growth arrest and apoptosis in target cells, 3) the anti-tumor effects of actinonin on AKR leukemias in AKR mice and 4) that these effects do not appear to be mediated through inhibition of CD13/APN.

Actinonin had significant anti-proliferative effects on human leukemia cells of various derivations. The cytotoxicity of actinonin was directly determined by trypan blue analysis and [^{3}H]leucine incorporation. The IC_{50} was about 2 to 5 μg/ml (Table 1). However, the IC_{50} for other CD13/APN inhibitors, amastatin and bestatin, was above 100 μg/ml (data not shown). Actinonin not only inhibited growth of CD13 positive cells (NB4 or HL60 cells) but also CD13 negative cells (RAJI or DAUDI cells) (Table 1), suggesting that the effect is not mediated by CD13/APN. Experiments with monoclonal antibody (mAb) F23 also suggested that the effect was not mediated by CD13/APN as cell viability was not changed by treatment with mAb F23 in comparison to no treatment or treatment with an isotype matched control antibody. In addition, the inhibitory effect of actinonin on CD13 positive cells was not blocked by pretreatment with the anti-CD13/APN mAb F23 (20). Since actinonin binds to F23 epitopes and is blocked by prior

incubation with mAb F23 (18) and mAb F23 is not inhibitory of cell growth, it was concluded that actinonin induced cell death in human leukemia cells is not likely to be associated with binding and inhibition of cell surface CD13/APN.

The effect of actinonin *in vitro* was mediated at least partly through G1 arrest and apoptosis. After 10 μg/ml actinonin treatment for 96 hours, 20-35% of NB4 and HL60 cells showed evidence of apoptosis (Table 2). 1,10-phenanthroline, which inhibits APN activity by chelating the zinc ion (3, 21), also induces apoptosis in HL60 cells (22). Actinonin binds to zinc domains of CD13/APN as determined by competition assays (18). This may suggest that the zinc binding motif of important intracellular enzymes may account for apoptosis, but cell surface CD13/APN does not appear to be associated with actinonin action. In addition, there is no increase in the percentage of CD11b in NB4, HL60 or RAJI cells (data not shown) after actinonin treatment, suggesting that there is no differentiating activity induced by actinonin.

Actinonin was also cytotoxic to RAJI cells, without an increase in apoptosis, in preliminary experiments designed to screen for other mechanisms or pathways involved in the anti-proliferative effects in these cells, BCL-2 expression was studied. BCL-2 expression, observed by intracellular flow cytometry in about 20% of HL60 cells and 60% of NB4 cells, showed no significant changes after treatment with actinonin (Table 4). BCL-2 expression, however, was decreased by 80% in RAJI cells after treatment with actinonin. Other cell surface proteins (CD11b, 13, 15, 29, 33, HLA-A) were significantly

decreased in NB4 cells after treatment with actinonin, consistent with the toxic effects of actinonin (Table 5).

Bestatin, another APN inhibitor, has shown anti-tumor therapeutic effects in several clinical trials (23). In a multi-institutional study, 101 patients with acute nonlymphocytic leukemia (ANLL) were randomized to receive bestatin or control. The bestatin group achieved a statistically significant prolongation of both the remission duration and survival in patients aged 50 to 65 years (24). It also normalized the CD4/CD8 ratio in peripheral blood and maintained the immune homeostasis in cancer patients (25, 26). This may be beneficial to AIDS patients with lymphoma. Recently, bestatin also showed direct inhibition of the growth of human choriocarcinoma in nude mice in a dose dependent manner (27). A recent randomized study in patients with non-Hodgkins lymphoma after autologous bone marrow transplant who were treated with bestatin showed increases in NK activity in pokeweed mitogen and phytohemagglutinin (PHA) responses of lymphocytes, in CD4 T cell and B cell numbers (26). Results with bastatin prompted the investigation whether a more potent APN inhibitor, actinonin, could inhibit the growth of syngeneic AKR leukemia cells in a mouse model *in vivo*. Actinonin showed dose dependent anti-tumor effects on AKR leukemia *in vivo*. There was a significant effect at doses of 100 µg per mice (5 mg/kg). Prolonged treatment appeared to further improve the activity against the leukemias. Since actinonin shows no apparent toxicity to mice in doses up to 400 mg/kg in mice (about 8 mg per mouse) (16), and no

apparent toxicity was seen in the experiments conducted here, the drug may be considered safe in this mouse model at these doses. In contrast, little anti-tumor activity was seen in a nude mouse model. One explanation for this may be related to the hypothesis that these inhibitors work via a nonspecific immune augmentation *in vivo* (26, 28) which may not be possible in nude mice. Alternatively, there may simply be differences in the biology of the cells that account for the lack of effects with RAJI cells.

In conclusion, the data showed that actinonin induces G1 phase arrest and apoptosis in human leukemia and lymphoma cells; moreover, actinonin can treat AKR leukemia in AKR mice with minimal toxicity. The site of action does not appear to be via inhibition of CD13/APN.

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Any patents or publications mentioned in this

specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A method of treating a neoplastic cell comprising the step of administering a pharmacologically effective dose of actinonin to said cell.

2. The method of claim 1, wherein said neoplastic cell is selected from the group consisting of lymphomas, leukemias, carinomas, sarcomas, and other pathological states in humans involving lymphocytes.

3. The method of claim 1, wherein said neoplastic cell is in a human.

4. The method of treating of claim 1, wherein said neoplastic cell is *in vitro*.

5. The method of claim 1, wherein actinonin is administered to an individual at the concentration of about 0.1 mg/kg to about 100 mg/kg.

6. The method of claim 1, wherein said actinonin induces cell growth arrest and apoptosis.

7. A method of treating an individual having a lymphoma, comprising the step of administering a therapeutically effective dose of actinonin to said individual.

8. The method of claim 7, wherein actinonin is administered to said individual at the concentration of about 0.1 mg/kg to about 100 mg/kg.

9. A method of treating an individual having a leukemia, comprising the step of administering a therapeutically effective dose of actinonin to said individual.

10. The method of claim 9, wherein actinonin is administered to said individual at the concentration of about 0.1 mg/kg to about 100 mg/kg.

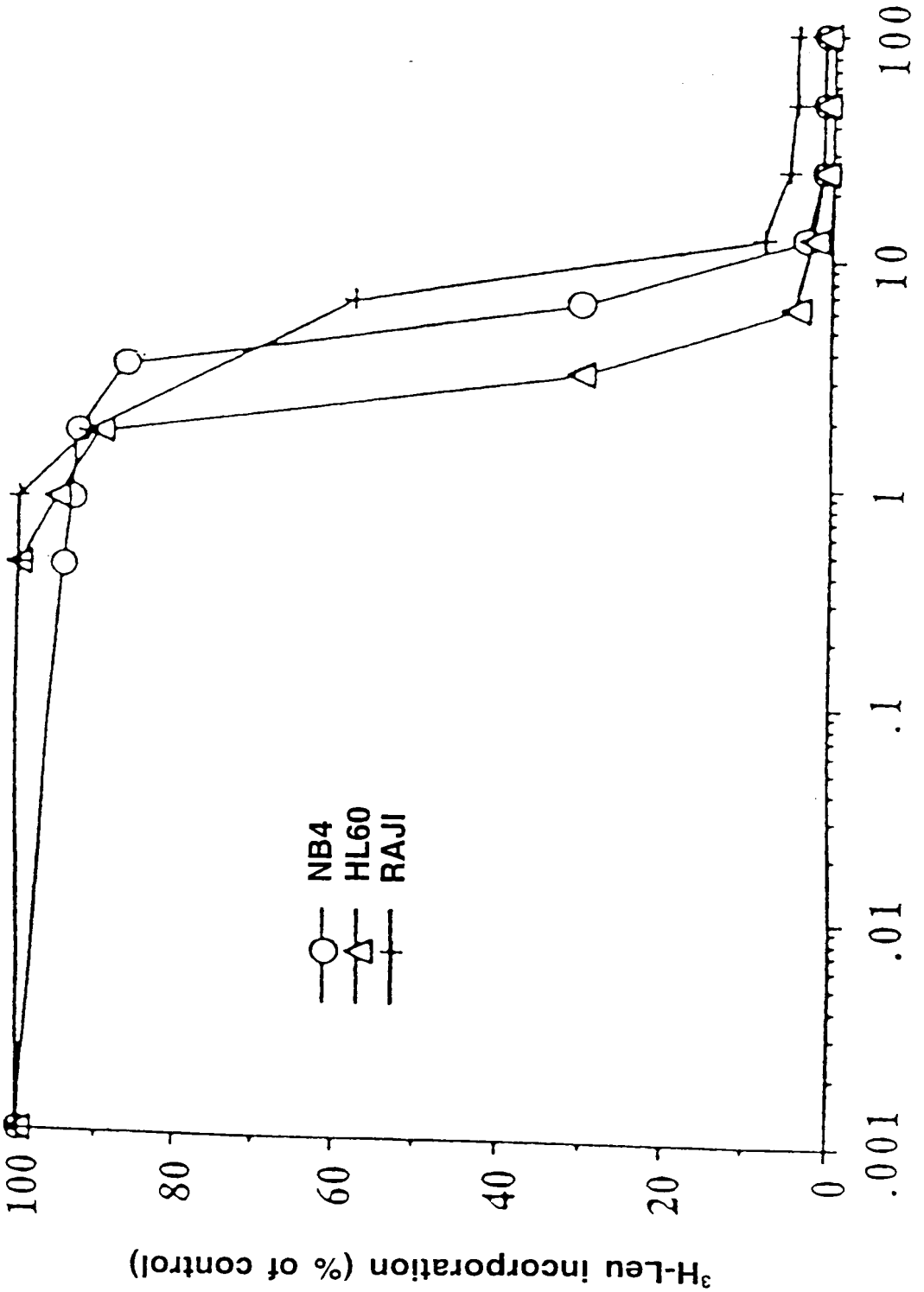

FIGURE 1A

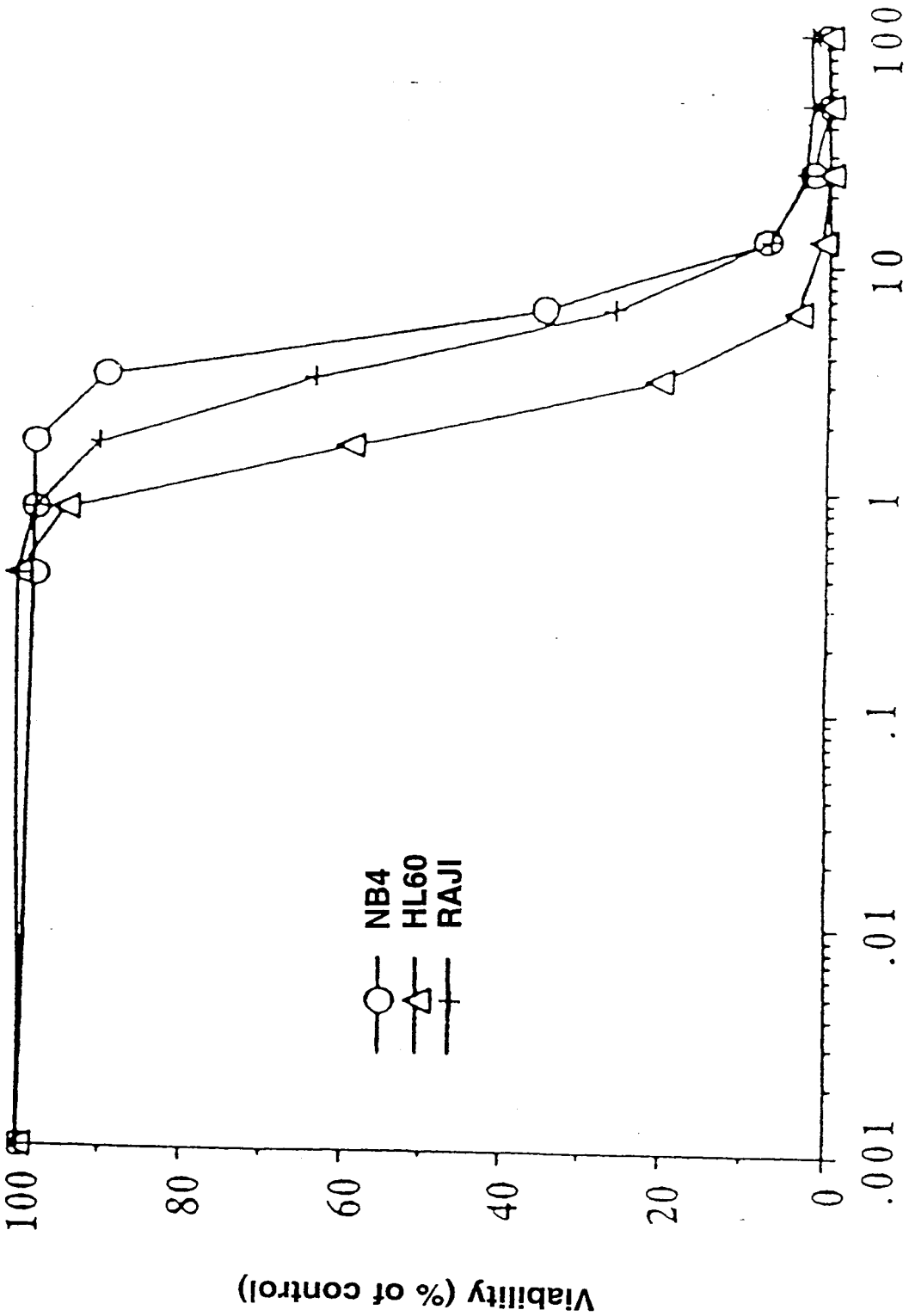
Viability (% of control)
100
80
60
40
20
0
.001
.01
.1
1
10
100
Actinonin (μg/ml)
NB4
HL60
RAJI

FIGURE 1B

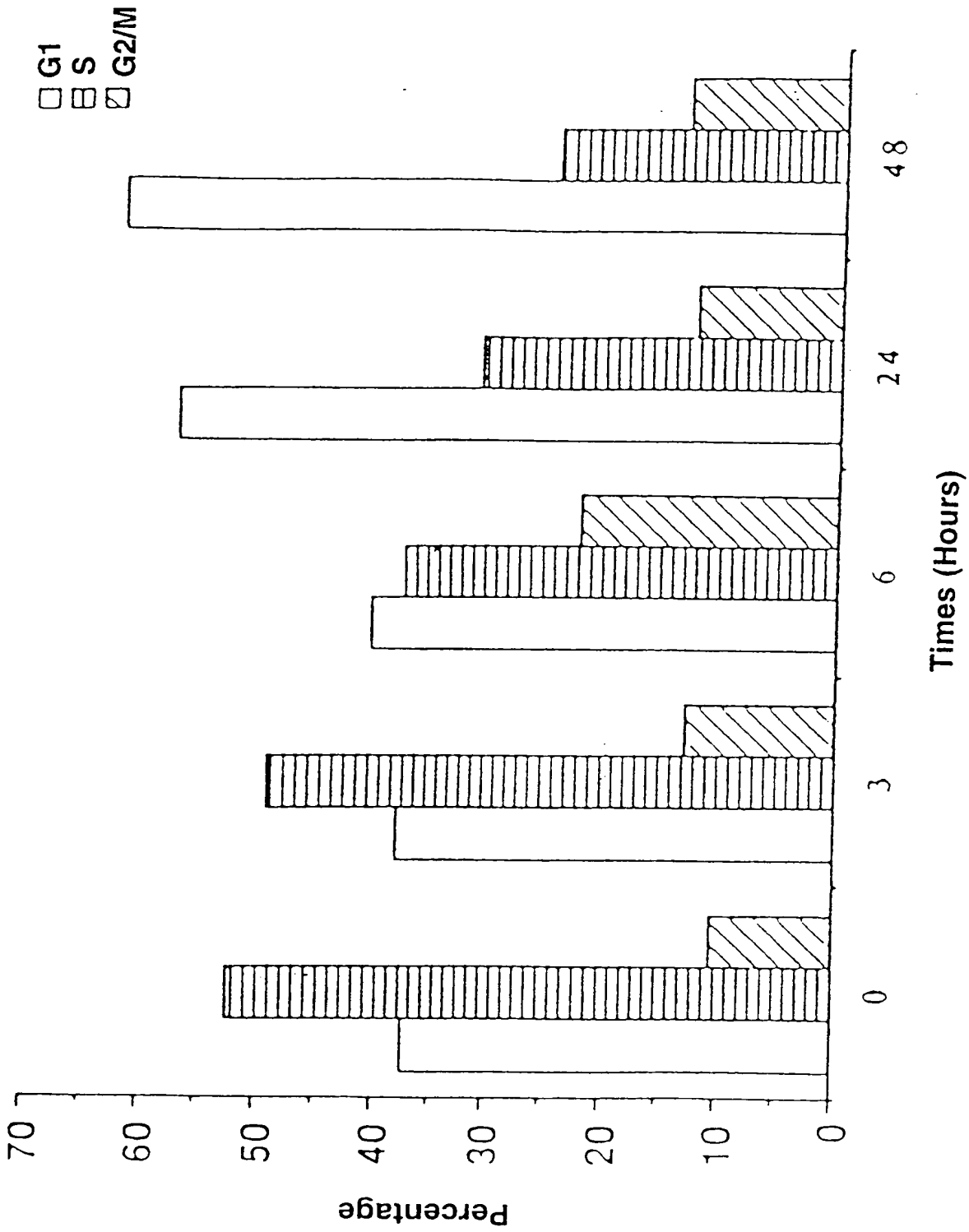

FIGURE 2

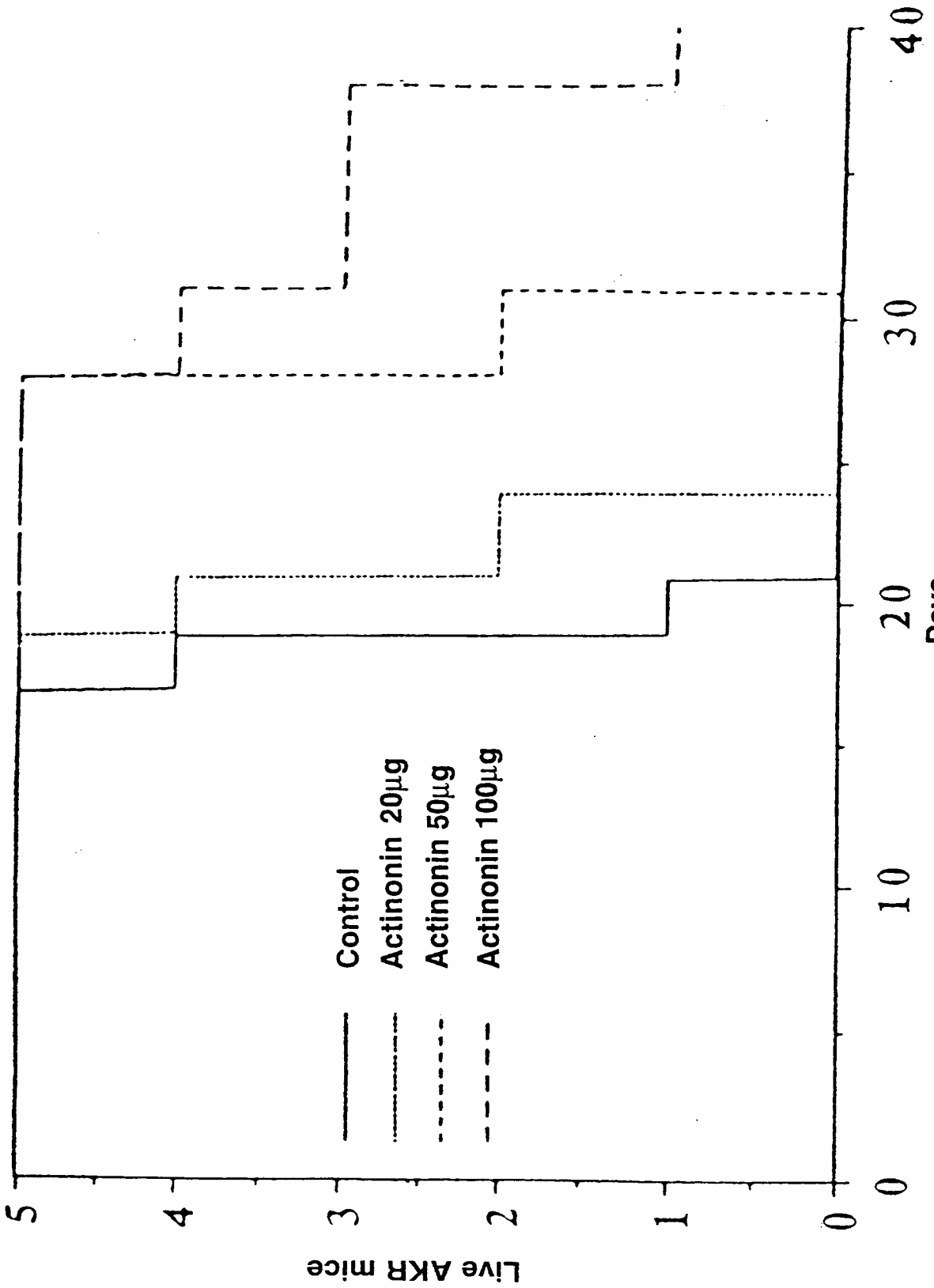
Live AKR mice
0
1
2
3
4
5
0
10
20
30
40
Days
Control
Actinonin 20μg
Actinonin 50μg
Actinonin 100μg

FIGURE 3A

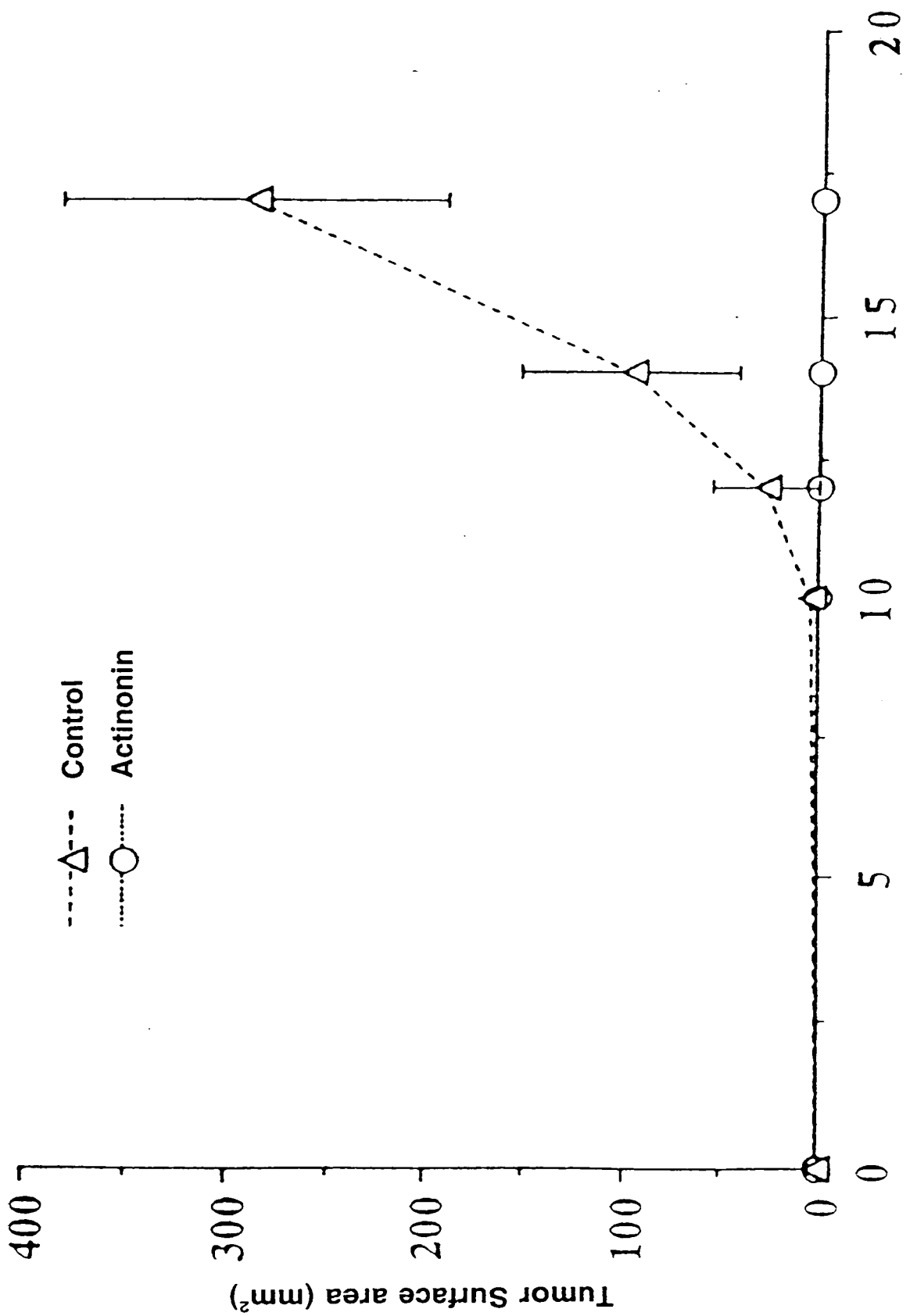

FIGURE 3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06363

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/40
US CL :514/423
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/423

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPATFULL- actinonin for the treatment of neoplastic disease, tumors or, cancers

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,663,342 A (UMEZAWA et al.) 05 May 1987, column 5.	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JUNE 1998	Date of mailing of the international search report 19 AUG 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer M. MOEZIE Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

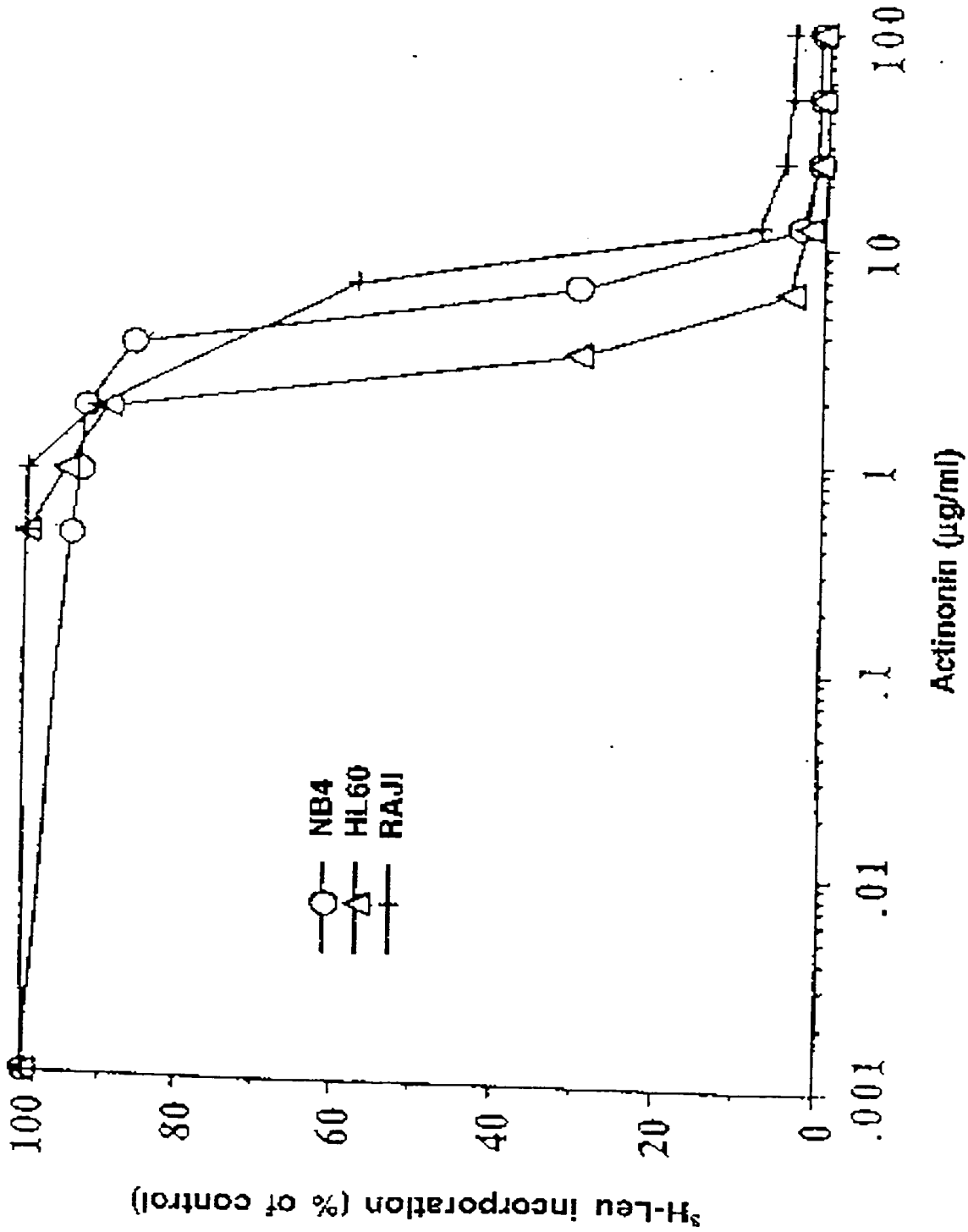

FIGURE 1A

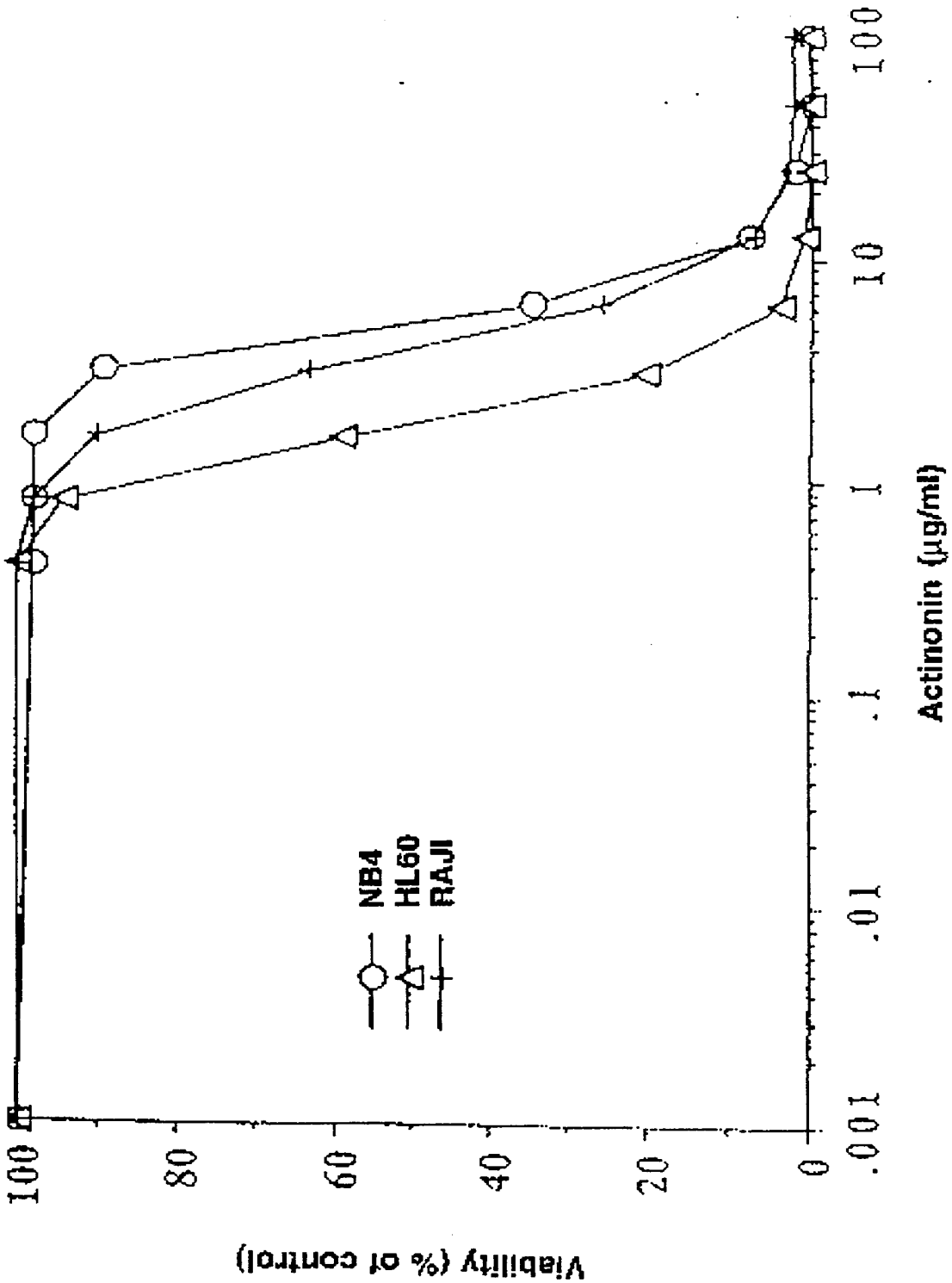

FIGURE 1B

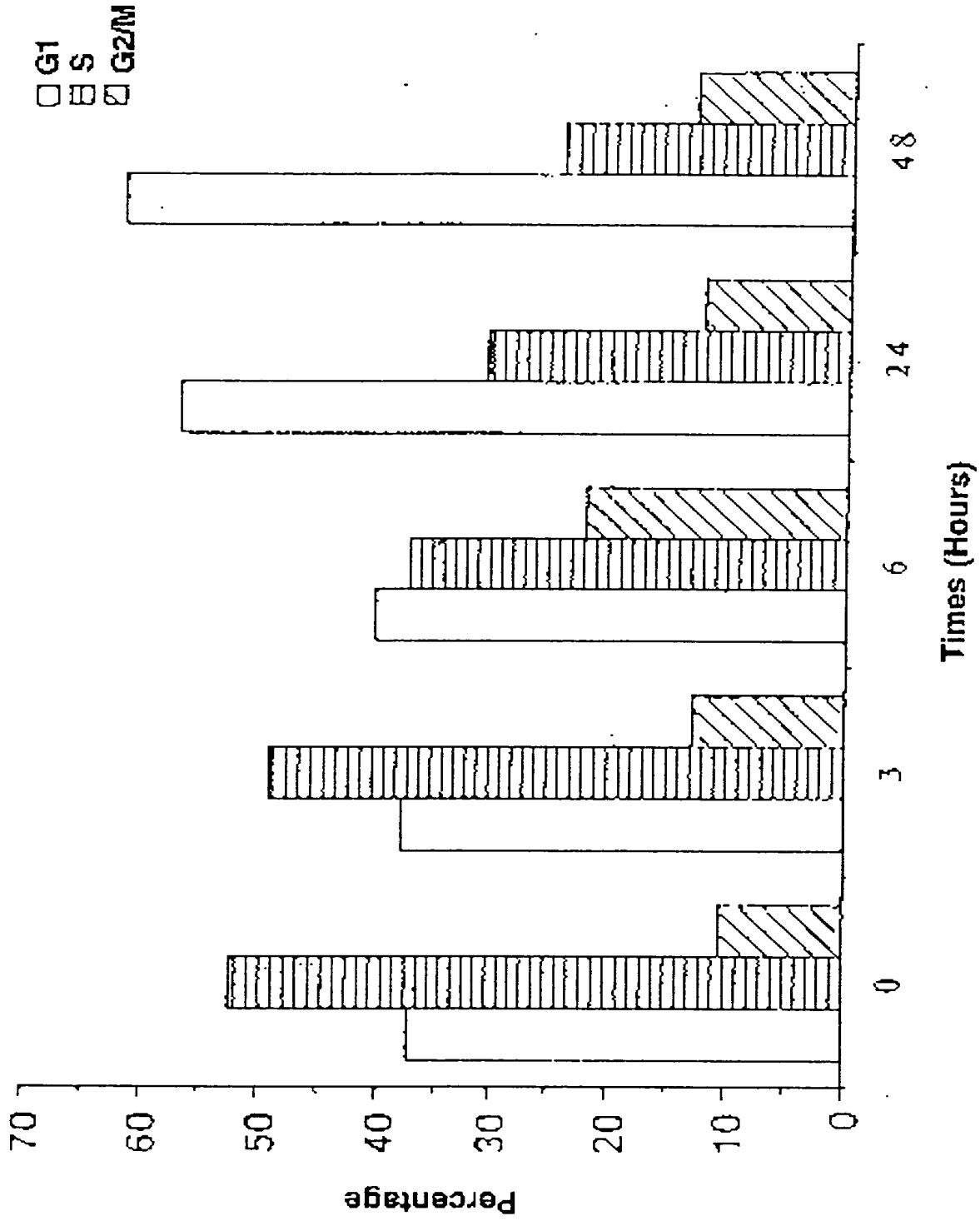

FIGURE 2

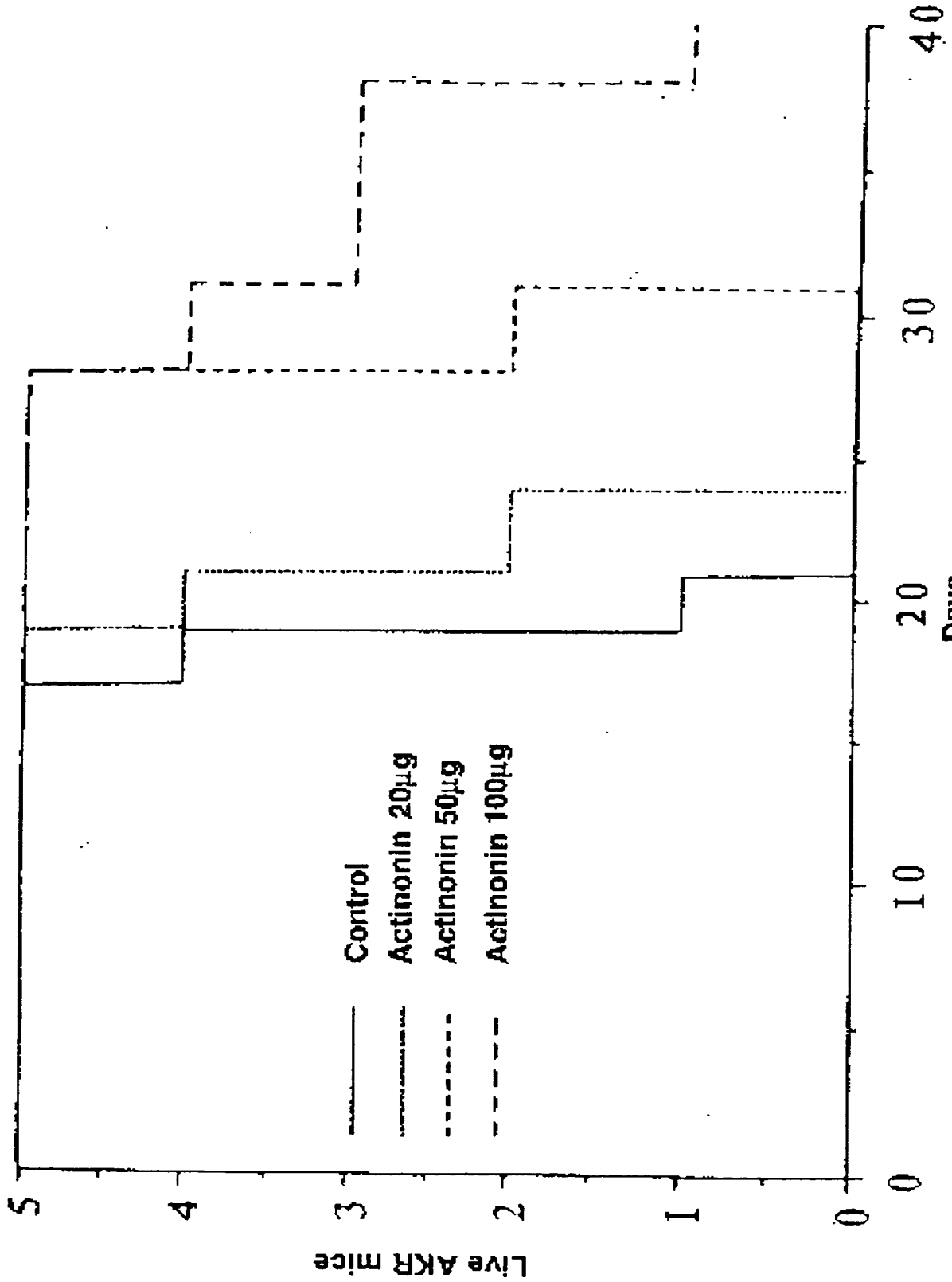

FIGURE 3A

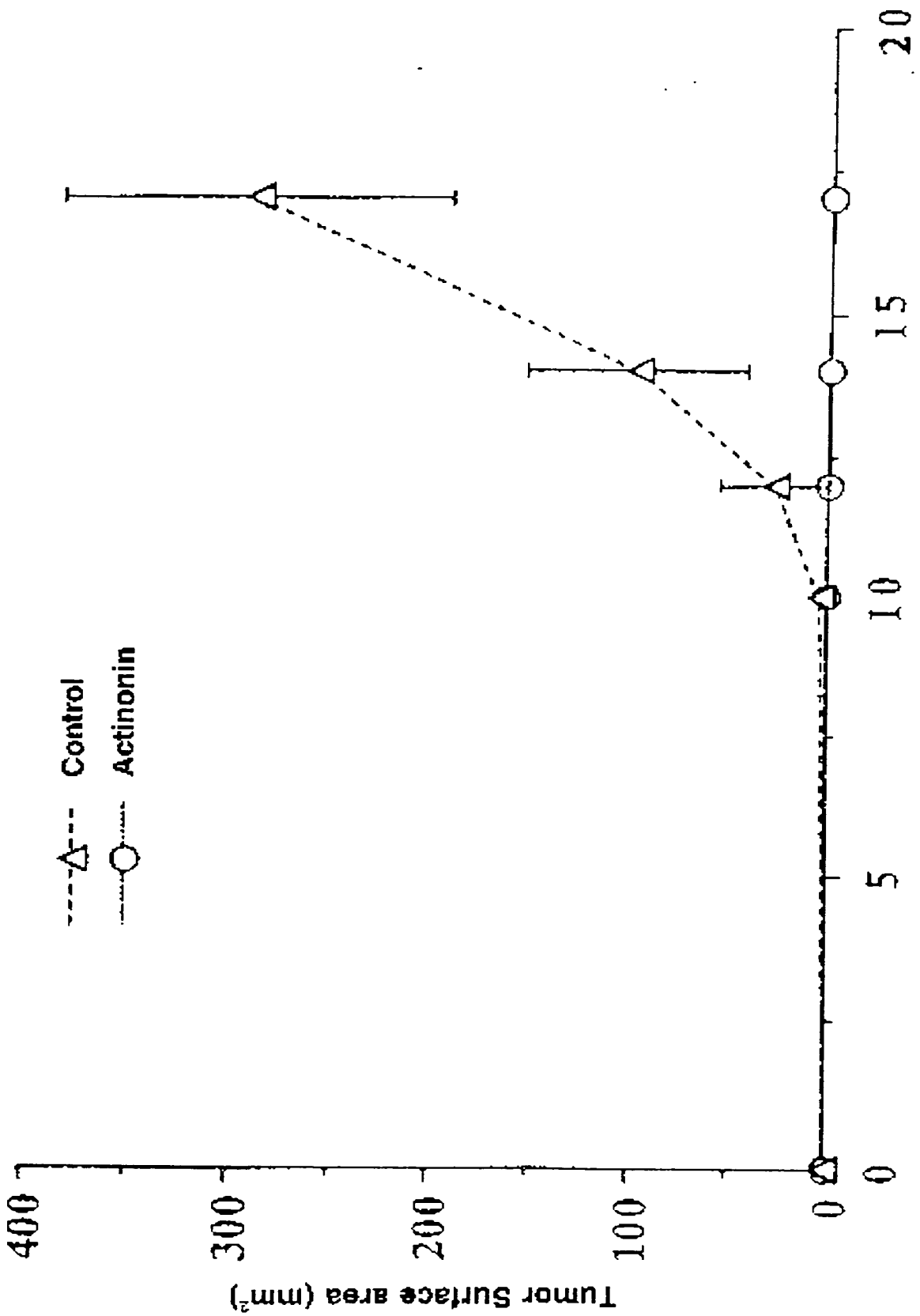

FIGURE 3B